

(19) FRENCH REPUBLIC
NATIONAL INSTITUTE OF INDUSTRIAL PROPERTY
PARIS

(11) Publication No.: **2 686 896**
(use only to order copies)

(21) National Registration No.: **92 01128**

(51) Int Cl⁵: C 12 N 1/20, 1/21, A 61 K 39/02

(12) **PATENT APPLICATION A1**

(22) Filing Date: 31 Jan 92

(30) Priority:

(43) Date Application Laid Public: 6 Aug 93, Bulletin 93/31

(56) List of Documents Cited in the Search Report: See the end of the present document.

(60) Reference to Other Related National Documents:

(71) Applicant(s): PASTEUR INSTITUTE - FR

(72) Inventor(s): Cossart Pascale, Kocks Christine, Goossens Pierre and Milon Genevieve

(73) Proprietor(s):

(74) Attorney: Cabinet Lavoix

(54) **Attenuated Mutant of Listeria Monocytogenes; Recombinant Strain of Listeria Monocytogenes, Use as Heterologous Vectors of Vaccinal Antigens and Use as Vaccine or Diagnostic Composition**

(57) The subject of the invention is an attenuated mutant of Listeria monocytogenes comprising, in the act A gene or in the promotor of the latter, a mutant able to block or modify the expression of the protein encoded by the act A gene.

This mutant can be used as a live vector for the expression of a heterologous DNA, in particular, a gene encoding for a viral, bacterial or parasitic antigen protecting the target of T-lymphocytes of subcategory CD8.

The recombinant mutant strains thus obtained have applications as a vaccine or diagnostic composition for testing the degree of protection of a host.

The present invention concerns an attenuated mutant strain of *Listeria monocytogenes* and its immunotherapeutic and diagnostic applications, in particular for the construction of a recombinant strain which can be used as a vaccine.

Listeria monocytogenes is a facultative aerobic bacillus, nonsporulating, Gram-positive, very widespread in the environment and responsible for human and animal Listeriosis. The disease is manifested by opportunistic infections, either by a meningitis and/or encephalitis, septicemia, or by miscarriage, with a high rate of mortality among newborns and adults whose defense mechanisms are weakened by pregnancy, a therapy-induced immune suppression, an underlying disease, or old age. Listeriosis can also affect apparently healthy persons.

Listeria monocytogenes is able to infect, in vivo or in vitro, a great variety of cell types, including the macrophages, the fibroblasts, the epithelial cells and the enterocytes.

After it penetrates into the infected cell, the bacteria lyses the membrane of the phagosome, using a hemolysin which it secretes. At the end of this phase, the bacteria is in the cytoplasm of the host cell.

Furthermore, *Listeria monocytogenes* is characterized by its ability to reproduce in tissues by direct infection from one cell to another, without leaving the cytoplasm (Racz, et al., 1970 (9)).

Shortly after its entry into the host cell, the bacteria surrounds itself with filamentous actin (F actin), which subsequently rearranges itself into a "comet" behind the bacteria in the direction opposite its movement (Tilney, et al., 1989, (1); Mounier, et al., (1990) (13). The polymerized actin is composed of short microfilaments, randomly oriented, differing from the long actin filaments usually observed in the muscle cells.

The bacteria are mobile and leave behind F actin "comets" several microns in length. Some of them are incorporated in cytoplasmic protuberances in the form of a finger, which can be internalized by the neighboring cells. The two plasma membranes surrounding the bacterium are then lysed. Once inside the cytoplasm of the new host cell, the bacterium can reproduce and begin a new cycle of dissemination.

During this dissemination process, the cells of *Listeria monocytogenes* are protected from the immune system of the host, and consequently the dissemination from one cell to another represents a key factor of the virulence.

By isolating and analyzing a mutant Tn 917-Lac unable to disseminate itself from one cell to another, the inventors have been able to identify a protein of *Listeria monocytogenes* involved in the actin assemblage caused by the bacterium.

The gene encoding for this protein, known as act A, is part of an operon (Mengaud, et al., 1991 (b) (8)) whose complete nucleotide sequence has recently been described (Vazquez-Boland, et al., 1992 (12)).

Thus, the subject of the present invention is a strain of *Listeria monocytogenes* of attenuated

virulence, characterized in that it comprises, in the act A gene or in the promotor of the latter, a mutation able to block or significantly modify the expression of the protein encoded by the act A gene.

The mutation can be produced by familiar techniques, in particular, by inserting into the act A gene or its promotor a sequence of one or more bases, preferably a stable transposon, deletion of one or more bases, mutations such as those caused by directed mutagenesis, for example, by PCR, and especially anti-sense mutations.

The mutation in particular can be accomplished by insertion of a transposon, such as the transposon Tn917-lac, as described in Mengaud, et al., 1991 (a) (7).

The mutation is preferably effected in the fragment of DNA encoding for the peptide sequence of repeating units contained between amino acids 235 to 315, 350 to 360, 367 to 385 and 389 to 393 of the peptide sequence SEQ ID No. 1.

Another advantageous mutation site, especially for insertion, is located downstream from the adenosine at position 497 of the nucleotide sequence of the act A gene.

This position corresponds to that between amino acids 61 and 62 of the peptide sequence SEQ ID No. 1.

A particularly preferred strain according to the present invention is the strain of *Listeria monocytogenes* LUT 12 deposited with the National Collection of Microorganism Cultures (CNCM) on 30 January 1992 as No. I-1167.

The mutants of the invention are able to confer on the hosts to which they are administered a protection against a subsequent infection by a pathogenic strain of *Listeria monocytogenes*.

Thus, another subject of the invention is a human or veterinary vaccine containing, as active ingredient, an attenuated strain of *Listeria monocytogenes*, as defined above.

This vaccine is able to confer an effective protection on man or animal, especially cattle and sheep, against listeriosis.

The immune response generated by the administering of an attenuated mutant as defined results in the proliferation essentially of T lymphocytes of subcategory CD8.

The T lymphocytes of subcategory CD8 are activated by peptides bound to antigens of MHC (major histocompatibility complex) Class I, which are generated by the proteolysis of proteins synthesized or liberated in the cytoplasm of cells presenting the antigen.

Thus, the attenuated mutants of *Listeria monocytogenes* according to the invention are able to stimulate the immune system by making use of the molecules of MHC Class I.

Consequently, by transforming *Listeria monocytogenes* with the help of an appropriate

plasmid, it is possible to introduce a heterologous gene coming from any given organism and to use the resulting recombinant strains as a system for expression of heterologous DNA.

Thus, another subject of the invention is a recombinant mutant of *Listeria monocytogenes*, characterized in that it contains a heterologous DNA either inserted into the genome of an attenuated mutant as defined above or carried by a plasmid replicating in the attenuated mutant.

The heterologous DNA preferably consists of a heterologous gene encoding for a protective antigen which is a target of T lymphocytes of subcategory CD8.

This antigen can be of bacterial origin (for example, mycobacteria), parasite origin (for example, *Leishmania*, *Trypanosoma* or *Toxoplasma*), or viral origin (influenza virus, lymphocyte choriomeningitis virus (LCMV), or the AIDS (HIV) virus).

A recombinant mutant of *Listeria monocytogenes* which is of particular interest contains the genes encoding for the gag antigen and/or the nef antigen of HIV or all or part of the envelope gp 120 of HIV 1 or gp 140 of HIV 2 or a peptide as defined in US-4 943 628.

The construction of the recombinant mutant can be carried out by transformation of an attenuated mutant as defined above, in particular, the mutant LUT 12, using an appropriate plasmid, and for example electroporation.

Advantageously, the cloning of the heterologous DNA will be done in *E. Coli* and one will employ a shuttle plasmid *E. Coli* - *Listeria monocytogenes* to accomplish the transformation.

As plasmids, one can mention pMKA (Sullivan, et al., (14)) or PHT 320 (Leredus, et al., (15)).

To enable the expression of the gene of interest (heterologous gene), it is advantageous to insert a strong promotor of *Listeria*, such as the hly promotor, upstream from the gene of interest.

In order for the product of translation of the gene of interest to be secreted, it is preferable to fuse the gene of interest at the start of hly, in order to use the "signal sequence" of listeriolysin O to liberate the protein of interest, encoded by the heterologous gene, in the cytoplasm of a host cell.

The recombinant strains of *Listeria monocytogenes* as defined above are advantageously suited to the preparation of a human or veterinary recombinant vaccine against an infection caused by a microorganism producing an antigen corresponding to the protein encoded by the heterologous DNA inserted into the genome of recombinant *Listeria monocytogenes*.

The vaccines according to the invention can be administered intravenously, subcutaneously, intramuscularly or orally.

An appropriate dose is between $5 \cdot 10^4$ and 10^9 cells/kg of weight.

This dose varies according to the route of administration, as well as the sensitivity of the

host.

The administration is preferably repeated so as to confer an effective protection on the host.

The recombinant mutants of *Listeria monocytogenes* as defined above are likewise suitable for the preparation of a diagnostic composition intended to test the state of protection of a human or animal host against an infection caused by a microorganism producing an antigen corresponding to the protein encoded by the heterologous DNA inserted in the genome of recombinant *Listeria monocytogenes* or expressed in this strain when carried by a plasmid.

It will be sufficient to inject the diagnostic composition of the invention locally, for example, subcutaneously, and observe after a certain latent period whether or not an inflammatory reaction occurs, such as the tuberculin test used to check the state of protection of a host against the tuberculosis bacillus.

We shall now describe how to obtain the mutant strain LUT 12 of *Listeria monocytogenes*, along with its properties, making reference to the enclosed figure:

A: the lecithinase operon of *Listeria monocytogenes* (Vazquez-Boland, et al., 1992 (12) with the position of the transposon in the mutant LUT 12 and the position of mutations by insertion of plasmids in the mutant.

B: the sequence of amino acids of the protein of the act A gene.

The black lines shown bold represent genes whose products have been characterized (mpl: metalloprotease, Domann, et al., 1991, (3) act A: (present invention), plc B: lecithinase) ORFX, ORFY and ORFZ are open reading frames.

P: indicates the promotor, the broken lines the transcription product, and Ω a potential transcription termination signal.

The potential signal sequence and the transmembrane segment are underlined. The region of repeating units is encircled. The arrow corresponds to the insertion of Tn 917-lac in the actA gene of the mutant LUT 12.

The numbering starts at the NH₂ end of the mature protein. The residues determined by microsequencing of the 90 kDa band are printed in bold and marked by an asterisk.

I - General DNA Cloning and Analysis Techniques

All the cloning and analysis techniques were carried out in keeping with standard protocols (Sambrook, et al., 1989 (10)) or according to the manufacturer's instructions.

The chromosomal DNA of *Listeria monocytogenes* was prepared as described by Mengaud, et al., 1991 (b) (8). The probes for Southern blot were prepared by PCR, purified from agarose gels by using the GeneClean kit (Bio 101, Inc., La Jolla, CA), and labeled by using the Multiprime

system of Amersham.

The Southern hybridizations were carried out with the rapid hybridization system (Amersham) on membranes of nylon N (Amersham) in a hybridization oven Hybaid.

II - Isolation of the LUT 12 Strain and Determination of the Point of Insertion of the Transposon

A bank of mutants Tn 917-lac, produced from the wild type strain L028 and the plasmid p TV32 carrying the transposon Tn 917-Lac, as described by Mengaud, et al., 1991 a (7), was screened on egg yolk gelose plates prepared from fresh egg yolk diluted at 1:2 in a solution of NaCl 150 mM, and adding 12.5 ml of this mixture to 250 ml of gelose with the addition of a brain-heart infusion (BHI) at 56°C.

A lecithinase-negative mutant not producing opacification of the egg yolk even after prolonged incubation and exhibiting a phenotype of wild type for all the other characters examined was designated LUT 12.

A - Biological Characteristics of this Mutant

This mutant had both a hemolytic activity and an in vitro growth rate identical to the wild type, yet proved to have a very attenuated virulence in the mouse.

TOXICITY

The LD₅₀ was higher by a factor 4 log 10 than the LD₅₀ of the wild type bacteria ($10^{8.55}$ bacteria instead of $10^{4.25}$).

TESTS FOR FORMATION OF PLAQUES ON FIBROBLAST CULTURES

Tests have been conducted on fibroblasts 3T3 (ECA CC88031146) by the technique described by Kuhn, et al., 1990 (5), except that the infections were produced by varying concentrations of inoculum: 1 to 25 µl of bacterial subcultures of 2 hours (A_{600nm} of 0.45), either nondiluted or diluted at 1/10.

This test shows the ability of *Listeria monocytogenes* to multiply in an intracellular fashion and to disseminate itself in single layers of fibroblasts covered with a layer of gelose containing gentamycine in a lethal concentration for the extra cellular bacteria but not for the intracellular bacteria. After several days, zones of dead cells destroyed by the bacterial infection are visible to the naked eye in the form of "plaques."

The mutant LUT 12 bacteria were unable to form plaques on single layers of 3T3 fibroblasts.

TEST FOR DISSEMINATION ON BONE MARROW MACROPHAGES

An observation under the optical microscope of the dissemination of *Listeria monocytogenes* in single layers of primary bone marrow macrophages was also performed as follows.

Suspensions containing macrophages were prepared from bone marrow of a female mouse C57BL/6, 7 weeks old, and cultivated in RPMI medium containing 10% fetal calf serum in the presence of supernatant L. 4.10^5 macrophages derived from bone marrow obtained on day 6 were inoculated on round glass plates (diameter 12 mm) the night before use. The macrophages were infected with an infection multiplicity (IM) of 0.04 (one bacterium for 25 macrophages, resulting in around 1% of cells infected, so as to be able to observe the individual points of infection caused by the progeny of a single bacterium. The infection was carried out as described for a macrophage J774.

After 30 minutes and after 8 hours, these cellular monolayers were fixed and stained with a Giemsa solution.

After 8 hours, the progeny of the wild type bacteria had been disseminated to numerous new host cells, and it was possible to observe bacteria having protuberances at their end. On the contrary, the progeny of mutant LUT 12 remained enclosed inside a single infected cell. The mutant bacteria either formed microcolonies or were disseminated in the cytoplasm of the host cells, but it was not possible to detect any protuberance containing bacteria.

This result shows that the mutant bacterium multiplies inside the infected cells, but is incapable of infecting adjacent cells by cell to cell dissemination.

TEST FOR GROWTH ON J774 MACROPHAGES

An experiment demonstrating that the mutant bacterium LUT 12 was able to multiply in an intracellular manner was carried out by means of a test for growth on J774 macrophages.

This experiment was carried out on single layers of J774 in plastic vials of tissue culture with 25 cm^3 volume. The cells were infected with an IM of 10 bacteria per cell. The number of intracellular bacteria was calculated after 2, 6 and 10 hours of growth on a medium containing gentamycine (5 $\mu\text{g/ml}$) by lysis of the cellular monolayers, washing with cold distilled water, and smearing of appropriate dilutions on plates containing a BHI medium.

After a period of 10 hours, the growth curves of the wild type and the LUT 12 bacteria were identical.

OBSERVATIONS UNDER THE ELECTRON MICROSCOPE

The intracellular behavior of the mutant LUT 12 was observed under the electron microscope. J774 macrophages were infected with wild type bacteria or the mutant strain for 30 minutes, followed by an incubation of 60 to 210 minutes in a medium containing gentamycine. For the mutant and the wild type, it was possible to observe free bacteria in the cytoplasm at $1\frac{1}{2}$ hours from infection. At this time, the wild type and the mutant were surrounded by a thin layer

of granular curly substance, but only the wild type had filamentous material assembled on its surface, comprising filaments of actin. At 4 hours from infection, the bacteria of wild type were surrounded by thick layers of F actin filaments. On the contrary, the LUT 12 mutant bacteria were almost bare. Even the fine curly coating observed at an early moment in the infection had disappeared.

In order to visualize the bacterial F actin association in a specific manner, the authors performed double fluorescence staining with the help of FITC-phalloidine, a fungal toxin binding to F actin, and with an anti-L. monocytogenes serum, followed by a second antibody coupled to rhodamine, in order to detect the bacteria. The J774 macrophages were infected for 4 hours with bacteria of wild type or mutant. While the bacteria of wild type were positively stained with FITC-phalloidine, the LUT 12 mutant bacteria though detectable with the anti-L. monocytogenes serum remained invisible with the actin stain.

These results show that the LUT 12 bacteria escape from the phagosomes as effectively as do the wild type bacteria and multiply within the cytoplasm. However, the mutant bacteria are never combined with actin F, they are incapable of movement inside the cell, and they cannot infect neighboring cells by direct dissemination. These observations suggest that the LUT 12 mutant lacks a necessary component for the process of formation of actin filaments induced by *Listeria monocytogenes*.

B - DETERMINATION OF THE POINT OF INSERTION OF THE TRANSPOSON

This mutant was analyzed by Southern blot to determine the number of transposons inserted in its chromosome.

The chromosomal DNA was digested by Bam HI, Eco RI, Hind III, Kpn I and Pst I.

Two different probes were used, corresponding to Tn 917-lac (Shaw and Clewell, 1985 (11)): one probe spanning 515 base pairs of the gene for resistance to erythromycin, obtained by PCR with the oligonucleotides 5'-TTG GAA CAG GTA AAG GGC ATT TAA-3' (position 821 to 824) and 5'-AGT AAA CAG TTG ACG ATA TTC TCG-3' (position 1313 to 1336), and a probe spanning the internal Hind III fragment of the transposon obtained by PCR with the oligonucleotides 5'-ACA ATT AAT GTC TCC CAT ATT-3' (position 3082 to 3102) and 5' (ACT GAT AAT TAA CCA AAA CAG-3' (position 4295-4315).

The transposon-chromosome junction was cloned from a chromosomal DNA bank obtained by restriction with Eco RI/Kpn I in pUC 18. A clone containing an insertion segment corresponding to the chromosome-transposon junction was isolated and sequenced directly from the plasmid by using an oligonucleotide hybridizing with the right end of the transposon (5'-CTA AAC ACT TAA GAG AAT TG-3', position 5244 to 5263).

The transposon was inserted after the adenine 497 in the nucleotide sequence of the fragment Hind III - Eco RI of the act A gene of the operon identified by Mengaud, et al., 1991 (b) (8), whose nucleotide sequence has been described by Vazquez-Boland, et al., 1992 (12).

The point of insertion of the transposon Tn 917-lac is shown in the diagram of Figure (A).

The lecithinase-negative phenotype of the LUT 12 mutant is probably due to a polar effect of the mutation by insertion in act A, inasmuch as the third gene of the operon plcB codes for lecithinase.

Supplemental studies were conducted and revealed that the loss of the actin polymerization activity was indeed due to a loss of expression of the gene act A.

Mutants were created by homologous recombination between the chromosome of *Listeria monocytogenes* and fragments corresponding to parts of the gene plcB and the open reading frames ORFX/Y and ORFZ (Figure (A)), situated downstream from the gene act A, by insertion of plasmids at various sites.

Immunofluorescence studies using FITC-phalloidine and rhodamine labeling of bacteria in infected J774 macrophages revealed that the mutants plcB, ORFX/Y and ORFZ were associated with actin F filaments, just like the bacteria of wild type. These studies were supplemented by electron microscope studies which revealed that these mutants were able to stimulate the assembly of actin in the same way as the wild type.

These analyses consequently show that mutations downstream from act A do not affect the assembly of actin A and suggest that the inability of mutant LUT 12 to polymerize cellular actin is due to absence of expression of the gene act A.

Furthermore, a transformation of mutant LUT 12 accomplished with act A shows that the wild type phenotype is restored, which rules out the possibility of a spontaneous mutation at another site of the chromosome.

These results also show that the product of gene act A is necessary to the assembly of the actin of *Listeria monocytogenes* and consequently for its pathogenic potency.

The product of gene act A has been determined as described hereafter.

III - Analysis of the Product of Gene act A

The nucleotide sequence of the gene act A suggests that it codes for a protein of 639 amino acids with one signal sequence and one transmembrane region (Vazquez-Boland, et al., 1992 120).

Supplemental studies were carried out on the one hand by comparative analysis of the surface proteins of *Listeria monocytogenes* of wild type and of strain LUT 12.

The bacterial isolates were grown in 200 ml of brain-heart infusion broth (BHI, DIFCO Laboratories, Detroit, Michigan), with the addition of erythromycin of 5 µg/ml for LUT 12, under agitation at 160 rpm on a Gyrotory G10 agitator (New Brunswick Scientific) at 37°C for 18 h.

The bacteria were harvested by centrifuge (5000 g for 20 minutes) and washed three times

in a saline solution of phosphate buffer (PBS).

The deposit obtained was put back into suspension in 4 ml of PBS, and SDS was added to a final concentration of 1%. At this concentration of SDS, the cells of *L. monocytogenes* do not lyse. The absence of bacteriolysis was verified under the microscope. After 5 minutes of agitation at room temperature, the bacteria were centrifuged (50,000 g for 10 minutes) and the supernatant was concentrated by ultrafiltration on microconcentrators (Centricom 30, Amicon) and kept at -20°C.

The protein concentration was determined by the bicinchoninic acid method (Pierce). The protein concentration was adjusted to 300 µg/ml for the electrophoresis. 10 µl of extract was mixed with 10 µl of buffer (SDS at 2%, glycerol at 10%, mercaptoethanol at 5%, bromophenol blue at 0.002% and Tris HCl 0.02 M), boiled for 3 minutes at 100°C. The electrophoresis was done at 60 mA for 120 minutes through discontinuous polyacrylamide gels (Laemmli, 1970 (6)). The bands were visualized by silver staining (Heukeshoven and Dernick, 1985 (4)).

For the marking of the cellular surface, 400 ml of an 18-hour culture of *L. monocytogenes* was centrifuged; the bacteria were washed 3 times with PBS at pH 7.4 and put back into suspension in 8 ml of PBS pH 8.0 at 4°C.

The bacteria were then treated with sulfosuccinimide biotin (sulfo-NHS-biotin; Pierce) at a final concentration of 0.5 mg/ml for 2 minutes under moderate agitation.

The cells were washed three times with PBS at pH 7.4 and extracted by SDS extraction.

The extracts corresponding to 7 µg of protein per channel were deposited onto SDS gels and treated as described by De Rycke, et al., 1989 (2) on nitrocellulose (BA 85, Schleicher and Schüll). The nitrocellulose filters were saturated for one night in PBS at 0.5% gelatin and incubated for 1.5 hours with streptavidin, conjugated to peroxidase (Jackson) in PBS containing 0.5% gelatin and 0.1 M of Tween 20. After various washings in the same buffer, the reaction bands were revealed with 0.5 mg/ml of 4-chloro-1-naphthol (Biorad) and 0.03% v/v of H₂O₂ in water.

The analyses of the electrophoresis gels reveal a band of 90 kDa for the wild type, which is absent from the LUT 12 strain. This band is likewise found in the *plcB* mutants and the LUT 12 mutants transformed by act A, as mentioned above.

The analyses of the surface marking by sulfosuccinimide-biotin directly reveal a biotinylated protein of 90 kA in the bacteria of wild type, which is absent from the mutant strain LUT 12.

In order to unambiguously identify the 90 kDa protein, the 90 kDa band was isolated and the sequence of 6 amino acids at the NH₂ end was determined and compared to the amino acid sequence deduced from the nucleotide sequence of gene *lac A*.

The extracts on SDS corresponding to 100 µg of protein per channel were brought to a boil in an SDS sampling buffer containing 7% (w/v) of urea before performing an electrophoresis on 7.5% SDS gels.

The proteins separated were transferred to a Problott membrane (Applied Biosystems) in Tris 50 mM - borate 50 mM for 17 hours at 4 to 5 V/cm. The proteins were stained for 5 seconds by means of 0.1% amido black in a 1% acetic acid and 40% methanol solution, and carefully rinsed with water. A 90 kDa band was cut out in several channels. The proteins of the membrane were sequenced by degradation per Edman in a 740 A sequencer of Applied Biosystems, with an HPLC PTH 120 A analyzer in line, programmed by the manufacturer for the Problott membrane. The amino acid sequences were analyzed on a Data General MV 10000 computer at the Scientific Information Processing Unit of the Pasteur Institute.

The sequence Ala-Thr-Asp-Sec-Glu-Asp of the isolated protein corresponds exactly to the amino acids of the cleavage site of the aforesaid signal sequence after the aforesaid peptide sequence starting at the gene act A (Figure B).

Consequently, the mature product of the gene act A is a protein of 610 amino acids with a molecular weight calculated at 67 kDa. It has an apparent molecular weight of 90 kDa and is expressed on the surface of the bacterium.

This protein is necessary to the assembly of actin F and its absence results in a very major attenuation of the virulence of *Listeria monocytogenes*. Consequently, any mutation affecting the gene act A or its promotor and significantly modifying or preventing the expression of its product will make it possible to obtain a nonpathogenic attenuated strain according to the invention.

Hereafter, we shall report on the results obtained in vivo with the LUT 12 strain involving the protection of mice against infection by *Listeria monocytogenes*.

IV - Effects in vivo for the LUT 12 Strain on the Mouse

The behavior of LUT 12 was studied after intravenous injection in the spleen and the liver of mice, which are the primary target organs where *L. monocytogenes* of wild type express their pathogenicity. The clinical assays used were as follows: the livers and spleens of infected mice were harvested at various times after infection and homogenized and dissected to enable the release of bacteria, and the living bacteria were counted in vitro. Thus, it was established that the number of LUT 12 in the spleen grew for the first 24 hours and decreased regularly until day 5.

In the liver, the number remained constant until day 4, at which time the elimination of the bacteria was highly effective (more than 3 log 10 bacteria are destroyed between day 4 and day 5).

Supplemental experiments revealed, furthermore, that mice previously infected with LUT 12 were resistant to a re-infection by the wild type LO28 of *L. monocytogenes*.

On the other hand, experiments were carried out to determine whether the harvested spleens of mice infected by LUT 12 contained T lymphocytes able to confer a protection on naive syngenic recipients against the virulent wild type of *L. monocytogenes*. The protection conferred was determined by counting the living *L. monocytogenes*, both in the livers and the spleens of the recipient mice, 48 hours after the infection. When they were harvested on day 7 after the infection

by the mutant strain LUT 12, the spleens contained cells able to protect the recipients of an infectious inoculum of the wild type of *L. monocytogenes*.

The cells conferring a protection were found to be among the T lymphocytes belonging to the subcategory CD8.

BIBLIOGRAPHIC REFERENCES

[in English]

THIS PAGE BLANK (USPTO)

LIST OF SEQUENCES

I - GENERAL INFORMATION

(1) APPLICANT: PASTEUR INSTITUTE

(2) TITLE OF THE INVENTION:

Attenuated Mutant of *Listeria monocytogenes*, Recombinant Strain of *Listeria monocytogenes*, Use as Heterologous Vectors of Vaccinal Antigen and Use as Vaccine or Diagnostic Composition.

(3) NUMBER OF SEQUENCES: 1

II - INFORMATION FOR SEQ ID No. 1

CHARACTERISTICS OF THE SEQUENCES

TYPE: protein

LENGTH: 610 amino acids

TYPE OF MOLECULE: surface protein

ORIGIN

ORGANISM: *Listeria monocytogenes*

CELL LINE: LO 28

CHARACTERISTIC

NAME OF THE PROTEIN: product of gene act A

III - DESCRIPTION OF THE SEQUENCE

[see original]

SYMBOLS OF THE AMINO ACIDS

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophane
Y	Tyr	tyrosine

CLAIMS

1. Attenuated mutant of *Listeria monocytogenes*, containing in the gene act A or in its promotor a mutation able to block or significantly modify the expression of the protein encoded by the gene act A.
2. Attenuated mutant of *Listeria monocytogenes* per Claim 1, characterized in that the mutation consists of an insertion, a deletion, or a mutation by directed mutagenesis.
3. Attenuated mutant of *Listeria monocytogenes* per Claim 1 or 2, characterized in that the mutation consists in the insertion of a stable transposon.
4. Attenuated mutant of *Listeria monocytogenes* per Claim 3, characterized in that the stable transposon is the transposon Tn917-lac.
5. Attenuated mutant of *Listeria monocytogenes* per any of the preceding claims, characterized in that the mutation is effected in the DNA fragment encoding for the peptide sequence with repeating units, contained between amino acids 235 to 315, 350 to 360, 367 to 385 and 389 to 393 of the sequence SEQ ID No. 1.
6. Attenuated mutant of *Listeria monocytogenes* per one of Claims 1 to 4, characterized in that the mutation consists in an insertion between the amino acids 61 and 62 of the peptide sequence SEQ ID No. 1.
7. Attenuated mutant of *Listeria monocytogenes* per Claim 6, designated LUT 12, deposited with the CNCM on 30 January 1992 as No. I-1167.
8. Human or veterinary vaccine, characterized in that it contains as the active component an attenuated mutant strain of *Listeria monocytogenes* according to one of the preceding claims.
9. Recombinant strain of *Listeria monocytogenes*, characterized in that it contains a heterologous DNA, either inserted in the genome of an attenuated mutant according to one of the preceding claims, or carried by a plasmid which replicates in the attenuated mutant.
10. Recombinant strain per Claim 9, characterized in that the heterologous DNA consists of a heterologous gene encoding for a protective antigen which is the target of T lymphocytes of subcategory CD8.
11. Recombinant strain per Claim 10, characterized in that the antigen is a bacterial antigen, especially one of mycobacteria.
12. Recombinant strain per Claim 10, characterized in that the antigen is a parasite antigen, especially of *Leishmania*, *Tripanosoma* or *Toxoplasma*.
13. Recombinant strain per Claim 10, characterized in that the antigen is a viral antigen, especially of HIV, lymphocyte choriomeningitis virus, or influenza virus.

14. Recombinant strain per Claim 13, characterized in that the antigen is the gag antigen and/or the nef antigen of HIV and/or all or part of the envelope gp 120 of HIV1 or gp 140 of HIV2.

15. Recombinant strain [per] one of Claims 9 to 14, characterized in that it contains a promotor of *Listeria* upstream from the heterologous DNA.

16. Recombinant strain per Claim 15, characterized in that the promotor is the promotor hly.

17. Recombinant strain per Claim 16, characterized in that the heterologous DNA is fused to the start of the hly gene, so as to use the signal sequence of listeriolysin O to secrete the product of the heterologous DNA in the cytoplasm of the host cell.

18. Human or veterinary recombinant vaccine, characterized in that it contains, as active component, a recombinant strain according to one of Claim 9 to 17.

19. Diagnostic composition containing a recombinant strain of *Listeria monocytogenes* according to one of Claims 10 to 17, for checking the state of protection of a human or animal host against an infection caused by a microorganism containing an antigen substantially identical to that encoded by the heterologous gene inserted in the recombinant mutant strain or carried by a plasmid replicating in the recombinant mutant strain.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.